



Cytotoxicity of epigallocatechin-3-gallate to LNCaP cells in the presence of Cu²⁺*

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Abstract: Epigallocatechin-3-gallate (EGCG) has shown remarkably anti-cancer activity, with its bioactivity being related to reactive conditions, such as pH and metal ions. The present study investigated the degradation of EGCG and its effect on prostate cancer cell in the presence of Cu²⁺. EGCG was incubated with prostate cancer cells, LNCaP, pretreated with or without Cu²⁺. EGCG in F-12 medium was quantified using HPLC and the viability of cells was assessed by gel electrophoresis, flow cytometry, and electron microscope. The results of HPLC showed that EGCG degraded completely within 12 h in F-12 medium with or without Cu²⁺. Gel electrophoresis and flow cytometry did not detect apoptosis of LNCaP cells when they were incubated with EGCG. Electron microscopy examination revealed that EGCG-Cu²⁺ complex led to damage of cytoplasm membrane in LNCaP cells. It was speculated that not EGCG, but its oxide and complex with Cu²⁺, are the bioactive components responsible for its cytotoxicity to LNCaP prostate cancer cells.

Key words: Copper, Epigallocatechin-3-gallate, LNCaP cells, Tea

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INTRODUCTION

Epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea, showed remarkably anti-inflammatory and anti-cancer activity in many animal tumor models, cell culture systems, and epidemiological studies (Liao *et al.*, 1995; Chung *et al.*, 2001a; 2001b; Kenshi *et al.*, 1997; Yang *et al.*, 1999; Lu *et al.*, 2002). Many reports proved the structure-activity relationships (Cutter *et al.*, 2001; Nihal *et al.*, 2000; Hiipakka *et al.*, 2002) of EGCG in the biological system. It is known that the structure of EGCG would quickly change in some conditions and that the structural rearrangement would easily occur *in vivo*. Previous studies investigated the metabolism of EGCG *in vivo* and suggested that the functional components are its metabolites but not EGCG itself (Unno *et al.*, 1996; Unno and Takeo, 1995; Nakagawa

et al., 1997).

Copper is one of the essential trace elements in humans and its daily requirement varies with ages. The recommended dietary allowance (RDA) for children is 200–400 µg/day while it is about 900 µg/day for adults. EGCG had been shown to chelate Cu²⁺ in various systems (Mira *et al.*, 2002; Kimura *et al.*, 1998; Yoshioka *et al.*, 2001; Kumamoto *et al.*, 2001); many previous studies used Cu²⁺ to initiate the free-radical oxidation of human low-density lipoproteins. To date, there are no reports of studies to examine the bioactivity of EGCG derivatives and EGCG-Cu²⁺ complex. The present study focused on cytotoxicity of EGCG-Cu²⁺ to human prostate cancer cell, LNCaP.

MATERIALS AND METHODS

Materials

EGCG was obtained from Sigma (St. Louis, MO, USA); the human prostate cancer cell, LNCaP, was

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purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences; ANNEXIN V kit was obtained from Caltag Laboratories (California, USA). All other chemicals were extra-pure grade or analytical grade.

Cell culture

LNCaP cell line was cultured in F-12 medium (GIBCO, Invitrogen Corporation) supplemented with 10% fetal bovine serum and 100 U/ml penicillin-streptomycin. The cells were maintained at 37 °C and 5% CO₂ in a humid environment (Shellab, USA).

HPLC analysis and EGCG content studies

Cells were seeded onto 25 cm² plates at a 1×10^5 density for 48 h, and incubated with different concentrations of EGCG and CuSO₄ (the concentration of CuSO₄ in F-12 medium was 0.00064 μmol/L). Then EGCG content was periodically determined in F-12 medium by HPLC (LC-2010A, Shimadzu, Japan) at 6, 12, 24 h. For the blank control, F-12 medium without cells was also incubated with different concentrations of EGCG and CuSO₄ for 6, 12, 24 h. The samples were analyzed on CLC-ODS column (Shim-pack, Shimadzu, Japan). A gradient elution was carried out in the following solvent systems: mobile phase A, double distilled water/acetonitrile (97:3, containing 0.5% acetic acid); mobile phase B, double distilled water/acetonitrile (70:30, containing 0.5% acetic acid). The linear gradient elution system was: from 100% A to 100% B in 45 min, standing at 100% B for 10 min and returning to 100% A after additional 5 min. The flow was set at 1.0 ml/min. Effluent was monitored at 280 nm (Shimadzu, Japan).

Gel electrophoresis and DNA fragmentation studies

Cells (1×10^5) were seeded onto 25 cm² plates for 48 h and incubated with different concentrations of EGCG and CuSO₄ for 24 h. Following this treatment, the cells were washed twice with pH 7.2 PBS (phosphate-buffered saline), followed by addition of 1 ml trypsinase and incubation for 5 min; then collected after centrifugation (300 g, 5 min) at 4 °C, and washed twice with pH 7.2 PBS. The cells were incubated with 200 μl cytolysis solution (0.1 mol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 25 mmol/L EDTA, pH 8.0, 0.5% SDS) and proteinase (50 μg/ml)

for 10 h at 50 °C. Then 500 μl ethanol was added into the pallets, and DNA was collected by centrifugation (12000 g, 10 min) at 4 °C and DNA was washed twice with 75% ethanol. After ethanol was volatilized entirely, the DNA was incubated with 30 μl TE (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and 4 μl RNase A (dissolved with TE, 50 μg/ml) for 1 h at 37 °C. The samples (10 μl) were mixed with 2 μl of loading solution (Bromophenol Blue 0.25%, xylene cyanol FF 0.25%, sucrose 40%), and were loaded onto a 1.2% (w/v) agarose gel (containing 0.5 μg/ml ethidium bromide). The DNA fragments were separated by electrophoresis (Amersham Biosciences) at 5 V/cm for 2.5–3 h in TAE buffer (40 mmol/L Tris-HCl, 20 mmol/L acetic acid, 1 mmol/L Na₂EDTA). The DNA was visualized by using imaging system (Tianneng Shanghai, China) and photographed by an Alphaimager 2000 digital camera.

Flow cytometry and cell damages studies

The cells were grown at a density of 2×10^5 in 50 cm² plates for 48 h, treated with different concentrations of EGCG and CuSO₄ for 24 h, washed with pH 7.2 PBS and trypsinized followed by centrifugation at 300 g for a period of 10 min, treated with ANNEXIN V kit and then analyzed by flow cytometry (FACS sort, BD, USA). The mortality and the apoptosis ratio of PCA cells were read in flow cytometry.

Detection of configuration by electron microscope

Cells were treated with different concentrations of EGCG and CuSO₄, and collected by centrifugation (300 g, 10 min). The samples were fixed overnight in 2.5% glutaraldehyde at 4 °C, buffered at pH 7.0 with phosphate buffer, washed three times, 15 min each time, then postfixed in 1% osmium tetroxide for 1 h, and washed three times with phosphate buffer (pH 7.0), 15 min each time, then dehydrated for 15 min each time, through a graded ethanol series (of 50%, 70%, 80%, 90%, 95%), followed by treatment with 100% ethanol and 100% acetone, 20 min each time. The samples were infiltrated for 1 h in epon-ethanol (1:1, v/v), and infiltrated 3 h in epon-ethanol (3:1, v/v). Then the samples were infiltrated overnight in epon, and the samples immersing in epon were placed into a 70 °C oven overnight. The samples were cut with a Du Pont diamond knife, and a Sorvall Porter-Blum MT2-B ultra-microtome. The samples were examined

with an electron microscope (JEM-1200EX, JEOL, Japan).

RESULTS AND DISCUSSION

Change of EGCG content in F-12 medium

EGCG of F-12 medium was quantified using HPLC. The ratio of EGCG to Cu^{2+} was chosen in the present study according to our previous study (Yu *et al.*, 2004). We found that EGCG declined rapidly at 37 °C in F-12 medium. After being incubated at 37 °C for 6 h, EGCG degraded from 10 $\mu\text{mol/L}$ to 0.63 $\mu\text{mol/L}$ when incubated with LNCaP cells. EGCG in F-12 medium in the absence of LNCaP cells was substantially lower than that in the presence of LNCaP cells. When the initial concentration of EGCG was

200 $\mu\text{mol/L}$, the degradation pattern was similar to that for 10 $\mu\text{mol/L}$. It was found that EGCG declined from 200 $\mu\text{mol/L}$ to 5.98 $\mu\text{mol/L}$ in the absence of LNCaP cells and to 9.64 $\mu\text{mol/L}$ when incubated with LNCaP cells.

Treatment of F-12 medium with Cu^{2+} caused a significant decrease in EGCG content. In the presence of Cu^{2+} , EGCG decreased substantially and was completely degraded at 6 h (Table 1).

Also, the ratio of EGCG to Cu^{2+} and the adding order are the factors in degradation of EGCG. At 12 h and 24 h, EGCG was not detected in all plates (Fig. 1) while EGCG was present in F-12 medium in a very small amount after 6 h under the present experimental conditions. The degradation products of EGCG remain unidentified. Probably, EGCG was oxidized, chelated and polymerized. As a good chelator, EGCG

Table 1 Concentration of EGCG in F-12 medium treated with different concentrations of EGCG and CuSO_4 ($n=5$)

Groups	Concentration of EGCG ($\mu\text{mol/L}$)					
	6 h		12 h		24 h	
	Without cells	With cells	Without cells	With cells	Without cells	With cells
10 $\mu\text{mol/L}$ EGCG	0	0.63 (± 0.1)**	0	0	0	0
200 $\mu\text{mol/L}$ EGCG	5.98 (± 0.9)	9.64 (± 1.2)*	0	0	0	0
320 $\mu\text{mol/L}$ CuSO_4 +200 $\mu\text{mol/L}$ EGCG	6.25 (± 0.8)	0**	0	0	0	0
200 $\mu\text{mol/L}$ EGCG+320 $\mu\text{mol/L}$ CuSO_4	0.99 (± 0.06)	0**	0	0	0	0
150 $\mu\text{mol/L}$ EGCG+32 $\mu\text{mol/L}$ CuSO_4	0.74 (± 0.03)	0**	0	0	0	0
32 $\mu\text{mol/L}$ CuSO_4 +150 $\mu\text{mol/L}$ EGCG	0	0	0	0	0	0

* $P < 0.05$, ** $P < 0.01$

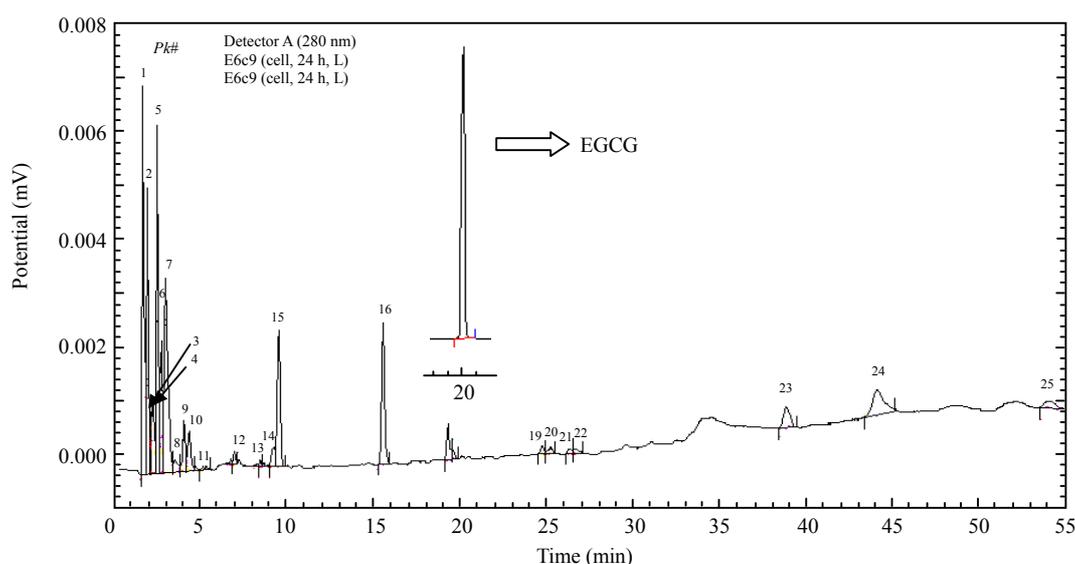


Fig. 1 HPLC chromatogram of F-12 with LNCaP cells incubated with 320 $\mu\text{mol/L}$ CuSO_4 pretreated with 200 $\mu\text{mol/L}$ EGCG for 24 h

may chelate with amino acid, protein, metal ion and so on. Hatano *et al.*(2003)'s study showed that albumin of fetal bovine serum chelated with EGCG in cell culture system. Probably, chelation of EGCG may be the main reaction existing in the system. It is speculated that interaction of EGCG or Cu^{2+} occurs when they are incubated with LNCaP cells.

Effects of EGCG on the growth of LNCaP cells

EGCG or Cu^{2+} showed dose-dependent suppression on growth of LNCaP cells. When CuSO_4 alone increased from 0.0064 to 320 $\mu\text{mol/L}$, the death rate of LNCaP cells increased from 21.9% to 79.11%. A similar trend was observed for EGCG when it was incubated with LNCaP cells in the absence of CuSO_4 (Table 2).

No synergistic effect was observed when EGCG and Cu^{2+} were incubated together with LNCaP cells. The sequence of adding EGCG and Cu^{2+} into the medium had significant impact on the mortality of LNCaP cells. As shown in Table 2, the death rate was 79.24% when 320 $\mu\text{mol/L}$ CuSO_4 was added into the medium followed by addition of 200 $\mu\text{mol/L}$ EGCG. In contrast, the death rate was only 38.5% when 200 $\mu\text{mol/L}$ EGCG was introduced into the medium first followed by addition of 320 $\mu\text{mol/L}$ CuSO_4 . The present results indicated that toxicity of EGCG to LNCaP cells was weakened in the presence of Cu^{2+} , and that interaction of EGCG with Cu^{2+} had a critical role in the system. In fact, our previous report demonstrated clearly that EGCG chelated strongly with Cu^{2+} , thus leading to a decrease in its toxicity to LNCaP cells. The results of gel electrophoresis and flow cytometry showed that the EGCG caused death of LNCaP cells were unlikely to be due to apoptosis (Fig.2, Fig.3).

Configuration of LNCaP cells detected by electron microscope

Ultrastructure of LNCaP cells was scanned by electron microscope. In the control culture, electron microscope revealed that LNCaP cells had intact cytoplasm membrane, clear cytoplasm, intact sub-cellular organelles, intact nuclear membrane and normal nucleolus (Fig.4a). In contrast, after treatment of LNCaP cells with 10 $\mu\text{mol/L}$ EGCG, the nuclear membrane and chromosome were intact but the cytoplasm became more vacuolated, and the cytoplasm membrane was damaged (Fig.4b). Similarly, exposure to 0.0064 $\mu\text{mol/L}$ Cu^{2+} led to damage of the cytoplasm membrane of LNCaP cells, but there was no significant change on organelle, nucleus, chromosome and cytoplasm (Fig.4c, Fig.4d). When LNCaP cells

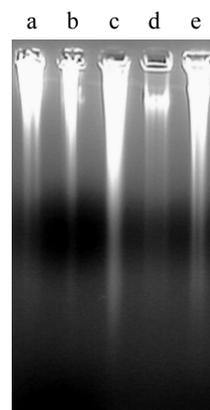


Fig.2 Gel electrophoretic analysis of DNA from LNCaP cells treated with EGCG and CuSO_4

a: LNCaP cells; b: LNCaP cells treated with 32 $\mu\text{mol/L}$ CuSO_4 before 150 $\mu\text{mol/L}$ EGCG was added; c: LNCaP cells incubated with 32 $\mu\text{mol/L}$ CuSO_4 after 150 $\mu\text{mol/L}$ EGCG was added; d: LNCaP cells treated with 200 $\mu\text{mol/L}$ EGCG after 320 $\mu\text{mol/L}$ CuSO_4 was added; e: LNCaP cells incubated with 320 $\mu\text{mol/L}$ CuSO_4 after 200 $\mu\text{mol/L}$ EGCG was added

Table 2 Death rate of LNCaP cells treated with different concentrations of EGCG and CuSO_4 (n=5)

Groups	Mortality of LNCaP cells (%)
0.0064 $\mu\text{mol/L}$ CuSO_4	21.9 (± 1.2)
64 $\mu\text{mol/L}$ CuSO_4	31.98 (± 1.0)
320 $\mu\text{mol/L}$ CuSO_4	79.11 (± 3.5)
50 $\mu\text{mol/L}$ EGCG	14.7 (± 0.9)
150 $\mu\text{mol/L}$ EGCG	62.61 (± 2.8)
200 $\mu\text{mol/L}$ EGCG	70.28 (± 5.0)
320 $\mu\text{mol/L}$ CuSO_4 +200 $\mu\text{mol/L}$ EGCG	79.24 (± 2.7)
200 $\mu\text{mol/L}$ EGCG+320 $\mu\text{mol/L}$ CuSO_4	38.51 (± 1.1)
150 $\mu\text{mol/L}$ EGCG+32 $\mu\text{mol/L}$ CuSO_4	68.13 (± 1.8)
32 $\mu\text{mol/L}$ CuSO_4 +150 $\mu\text{mol/L}$ EGCG	12.44 (± 0.5)

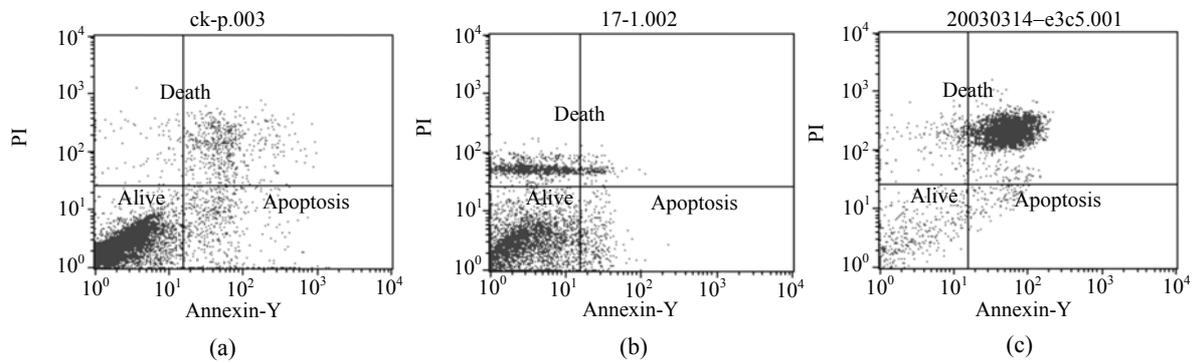


Fig.3 Detection of apoptosis by flow cytometry (partial figures are shown, all data are shown in Table 2). (a) Normal LNCaP cells; (b) LNCaP cells treated with 10 $\mu\text{mol/L}$ EGCG; (c) LNCaP cells incubated with 200 $\mu\text{mol/L}$ EGCG after 320 $\mu\text{mol/L}$ CuSO_4 was added

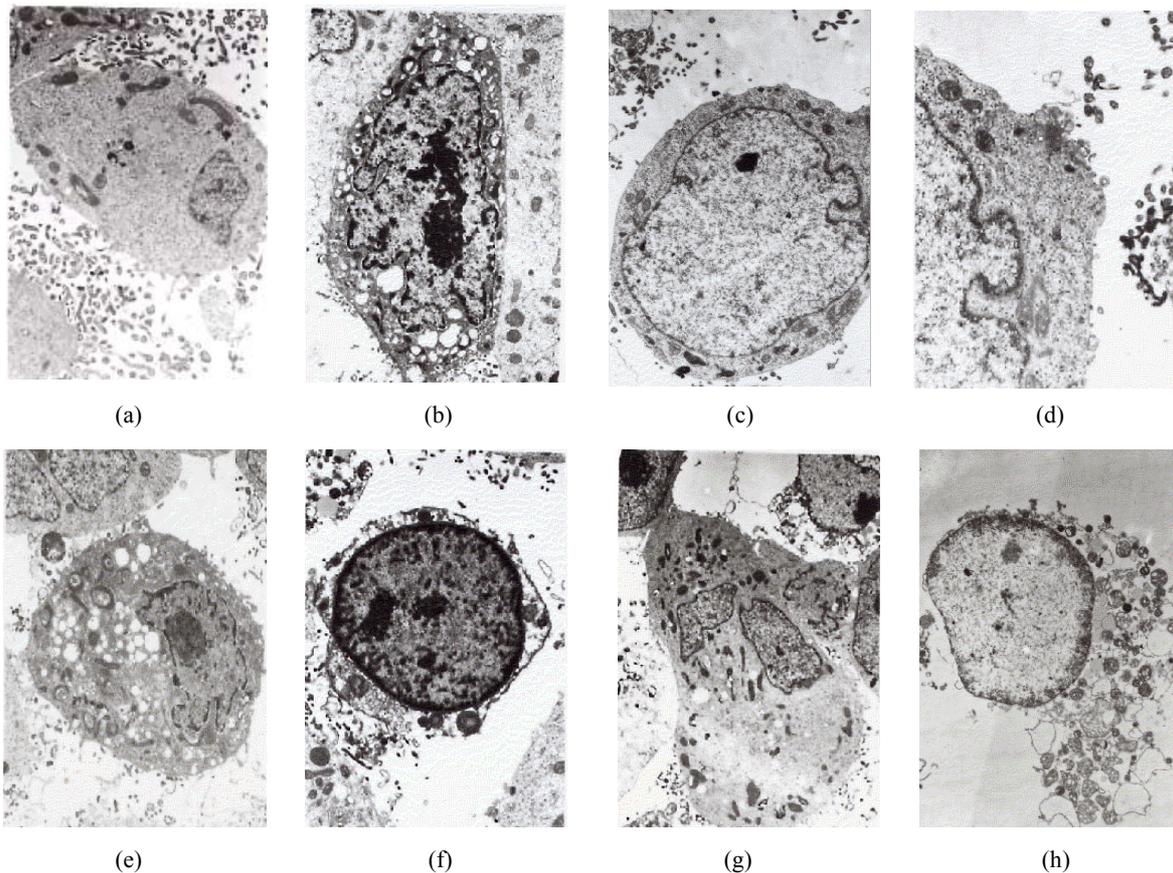


Fig.4 Configuration of LNCaP cells treated with different concentrations of EGCG and CuSO_4 . (a) Normal LNCaP cells; (b) Treated with 10 $\mu\text{mol/L}$ EGCG; (c) Treated with 0.0064 $\mu\text{mol/L}$ CuSO_4 ; (d) cytoplasm membrane of LNCaP cells treated with 0.0064 $\mu\text{mol/L}$ CuSO_4 ; (e) Treated with 150 $\mu\text{mol/L}$ EGCG in the presence of 32 $\mu\text{mol/L}$ CuSO_4 ; (f) Put into 150 $\mu\text{mol/L}$ EGCG, then added 32 $\mu\text{mol/L}$ CuSO_4 ; (g) Before addition of 200 $\mu\text{mol/L}$ EGCG, put into 320 $\mu\text{mol/L}$ CuSO_4 was added; (h) Treated with 320 $\mu\text{mol/L}$ CuSO_4 after 200 $\mu\text{mol/L}$ EGCG was added

were treated with 150 $\mu\text{mol/L}$ EGCG and 32 $\mu\text{mol/L}$ Cu^{2+} , the damage was similar to that of the cells incubated only with EGCG (Fig.4e). The cytoplasm membrane was almost completely destroyed when the cells were treated first with 150 $\mu\text{mol/L}$ EGCG and then with 32 $\mu\text{mol/L}$ Cu^{2+} . In this regard, cytoplasm and organelle were hardly detected at the same time while nucleus and chromosome were intact (Fig.4f). When 200 $\mu\text{mol/L}$ EGCG was added into the medium after addition of 320 $\mu\text{mol/L}$ CuSO_4 , karyokinesis of LNCaP cells was not inhibited, and nucleus and chromosome were not damaged (Fig.4g). There were many organelles observed in the cytoplasm, which was however, vacuolated; and the cytoplasm membrane was not intact. Furthermore, the cytoplasm membrane was destroyed severely when LNCaP cells were treated with 320 $\mu\text{mol/L}$ Cu^{2+} first and then with 200 $\mu\text{mol/L}$ EGCG (Fig.4h). The present results demonstrated clearly that toxicity of EGCG to LNCaP cells was associated with its damage to the cytoplasm membrane regardless of the presence or absence of Cu^{2+} .

There is no study to date examining the bioactivity of EGCG-copper complex and the effect of EGCG degradation compounds on cancer cells. EGCG has two rings and many hydroxyl groups, which can potentially participate in chelation with Cu^{2+} and be easily oxidized. As shown in this study, mortality of LNCaP cells was increased in the presence of Cu^{2+} , suggesting that EGCG-copper complex might be more toxic to LNCaP cells. As a matter of fact, it is difficult for EGCG to cross the cytoplasm membrane owing to its lipo-soluble character. The present results showed that changes of EGCG contents in F-12 medium without LNCaP cells were quite different from those in F-12 medium with LNCaP cells.

It is possible that EGCG had interacted with the cytoplasm membrane, and entered the cells. Electron microscopic observation revealed that the cytoplasm membrane was only damaged but that nucleus and organelle remained intact, demonstrating that the first target of EGCG and its derivatives to LNCaP cells was the cytoplasm membrane. This is in agreement with that of Hoshino *et al.* (1999) who found that the recycling redox reactions occurring between Cu^{2+} and Cu^+ in the microbe system involving catechins and hydrogen peroxide, caused the cytoplasm membrane

damages. Some previous studies showed that EGCG could induce apoptosis of cancer cells, including prostate cancer cell (Roy *et al.*, 2003; Paschka *et al.*, 1998; Gupta *et al.*, 2000; 2003; Liang *et al.*, 1997; Lin, 2002). This was in disagreement with the present observation. Probably, the discrepancy was due to different cells and incubation media and conditions. The present study is the first to examine the cytotoxicity of EGCG- Cu^{2+} complex on prostate cancer cells. It was concluded that cytotoxicity of EGCG was mediated by its damage to the cytoplasm membrane of LNCaP cells.

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